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Effects of ethanol and various alcoholic beverages on the formation of O^6-methyldeoxyguanosine from concurrently administered *N*-nitrosomethylbenzylamine in rats: a dose–response study

Y.Yamada[1], R.O.Weller[2], P.Kleihues[1] and B.I.Ludeke[3]

Institute of Neuropathology, Department of Pathology, University Hospital, CH-8091 Zürich, Switzerland

[1]Present address: 1st Department of Surgery, University of Tokyo, Tokyo 113, Japan

[2]Present address: Department of Pathology, Faculty of Medicine, Southampton General Hospital, Southampton SO9 4XY, UK

[3]To whom reprint requests should be sent

Consumption of alcoholic beverages has been identified as a major cause of oesophageal cancer in industrialized countries, with an exceptionally high risk associated with apple-based liquors (calvados). In the present study, we have determined the dose–activity relationship of the effects of coincident ethanol on the formation of O^6-methyldeoxyguanosine (O^6-MEdG) by the oesophageal carcinogen *N*-nitrosomethylbenzylamine (NMBzA). Male Fischer 344 rats received a single intragastric dose of NMBzA (2.5 mg/kg body wt; 7.4 ml/kg body wt) in tap water containing 0–20% ethanol (v/v). Survival time was 3 h. In controls, concentrations of O^6-MEdG were similar in oesophagus, lung and liver (11–14.9 μmol/mol dG). In oesophagus, coincident ethanol increased levels of O^6-MEdG from 15.2 μmol/mol (0.1% ethanol) to 46.0 μmol/mol (20%). This increase was dose dependent for 1–20% ethanol; however, low doses produced a larger effect per gram of ethanol than higher doses. In lung, concentrations of O^6-MEdG increased from 11 μmol/mol (0.1%) to a plateau value of 24 μmol/mol (≥5%). In nasal mucosa, an increase in O^6-MEdG from 3.9 μmol/mol (controls) to 30.7 μmol/mol was observed with 4% ethanol. Effects of ethanol on hepatic DNA methylation were statistically non-significant. Modulation of NMBzA bioactivation by various alcoholic beverages (adjusted to 4% ethanol) was also investigated. Increases in oesophageal O^6-MEdG were similar (+50% to +116%) with pear brandy, rice wine (sake), farm-made calvados, gin, Scotch whisky, white wine, Pilsner beer and aqueous ethanol. Significantly higher increases were elicited by commercially distilled calvados (+125%) and red burgundy (+162%). In contrast to its effects at an ethanol content of 4%, farm-made calvados diluted to 20% ethanol produced significantly higher (+200%) increases in oesophageal DNA methylation than aqueous ethanol (+148%). Our results show that ethanol is an effective modulator of nitrosamine bioactivation *in vivo* at intake levels equivalent to moderate social drinking, and that some alcoholic beverages contain congeners that amplify the effects of ethanol, suggesting that modulation of nitrosamine metabolism by acute ethanol may play a role in the etiology of human cancer.

*Abbreviations: O^6-MEdG, O^6-methyldeoxyguanosine; NMBzA, *N*-nitrosomethylbenzylamine.

Introduction

Consumption of alcoholic beverages and use of tobacco products are among the major causes of oesophageal cancer in industrialized countries (1). Epidemiological studies have demonstrated that though the effects of ethanol and tobacco are multiplicative (2,3), the relative risk for oesophageal neoplasms also increases progressively with daily intake of ethanol in non-smokers (2,4,5). Moreover, the risk for tumour development is dependent on the type of alcoholic beverage consumed and appears to be more pronounced for distilled spirits than for beer or wine (1,2,6). An exceptionally high risk has been inferred for cider and apple brandy (calvados), which are traditionally popular beverages in the French provinces of Brittany and Normandy where mortality rates for oesophageal cancer in males are elevated by 3- to 4-fold above the European average (1,2,7). While neither ethanol nor alcoholic beverages are carcinogenic *per se* (8–10), dietary ethanol has been observed to alter the organotropism and tumorigenic potency of a number of carcinogenic nitrosamines in laboratory rodents (9,11–16). Several studies have demonstrated that moderate amounts of concurrently administered ethanol inhibit hepatic nitrosamine metabolism, resulting in increased nitrosamine bioactivation in extrahepatic tissues in which monooxygenase activity either is not or is less sensitive to inhibition by ethanol (17–20). Nitrosamines, which are ubiquitous as environmental contaminants and as products of endogenous synthesis from ingested precursors, may constitute a substantial proportion of the total human carcinogen burden (21). An ethanol-mediated inter-organ shift in nitrosamine bioactivation could, therefore, significantly contribute to the enhancement associated with alcohol consumption of tumorigenesis in oesophagus and other organs in which malignant transformation in humans is otherwise less frequent (18).

In the present study, we have investigated the dose–activity relationship of modulation by ethanol of nitrosamine bioactivation. The effects of a single simultaneously administered dose of ethanol on the formation of the promutagenic base O^6-methyldeoxyguanosine (O^6-MEdG*) from *N*-nitrosomethylbenzylamine (NMBzA), a highly potent and selective oesophageal carcinogen (22), were assessed in oesophagus, lung and liver over a wide range of ethanol concentrations. In addition, we examined whether alcoholic beverages, including commercialy distilled and farm-made calvados, exert a shift in NMBzA bioactivation which exceeds that of a similar dose of ethanol alone.

Materials and methods

Chemicals and antibodies

RNase T_1 from *Aspergillus oryzae* was purchased from Boehringer-Mannheim (Schweiz) AG, Rotkreuz, Switzerland. RNase A from bovine pancreas and calf thymus DNA were purchased from Sigma Chemie, D-8024 Deisenhofen, Germany. DNA purification-grade lysis buffer and 70% phenol/water/chloroform reagents were from Applied Biosystems, Inc., Foster City, CA, USA. Characteristics of the rabbit antiserum raised against keyhole limpet hemocyanin conjugates of O^6-methylguanosine (NPZ 193–1) have been described previously

(23). Goat anti-rabbit IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolylphosphate toluidine salt and nitroblue tetrazolium chloride were obtained from BioRad, Glattbrugg, Switzerland. All commercial chemicals were of analytical grade or higher.

Alcoholic beverages

Farm-made calvados was kindly provided by Dr A.M.Mandard, Centre François-Baclesse, Caen, France. All other alcoholic beverages were from commercial sources. Ethanol concentrations of the undiluted beverages (Table I) and of all dosing solutions were determined with a kit (cat. no. 176290) purchased from Boehringer-Mannheim (Schweiz) according to the manufacturer's directions.

Animals and treatment

Male Fischer 344 rats (100–160 g) were obtained from Charles River Wiga GmbH, Kisslegg, Germany and maintained on a standard laboratory diet with water *ad libitum*. NMBzA was administered as a single dose of 2.5 mg/kg (18 μmol/kg) by gavage in a volume of 7.4 ml/kg body wt. For the determination of the dose–activity relationship of the effect of ethanol on DNA methylation by NMBzA, the carcinogen was dissolved in tap water containing 0, 0.1, 0.5, 1, 5, 10 or 20% ethanol (v/v). Five animals were treated with each ethanol concentration. In a second experiment, groups of five animals received NMBzA dissolved in water, 4% aqueous ethanol, or one of nine alcoholic beverages (Table I). The ethanol content of the beverages was adjusted to a final concentration of 4% with tap water prior to adding the nitrosamine. After a survival time of 3 h, the animals were killed by exsanguination during ether anaesthesia. Tissues were removed rapidly, frozen in liquid nitrogen and stored at −70°C until analysis.

DNA isolation

DNA was isolated by automated phenolic extraction using a Model 340A Nucleic Acid Extractor (Applied Biosystems, Inc.). Liver tissue (0.3–0.5 g) was pulverized in liquid nitrogen, homogenized in 10 ml of PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) with 10–12 passages in a Potter–Elvehjem homogenizer and filtered through a single layer of nylon gauze (30 μm mesh; Willi Fischer Labortechnik, Frankfurt/Main, Germany). Cell nuclei were collected by centrifugation for 5 min at 1000 *g*, resuspended in 0.2–0.4 ml of PBS and transferred to 5 ml Potter–Elvehjem homogenization vessels. Following the addition of 2 ml of lysis buffer, the mixtures were homogenized by hand (three passages) and transferred to 3 ml of lysis buffer. Crushed oesophagi and nasal cavity scrapings (0.3 g) were homogenized directly in 5 ml of lysis buffer and filtered through nylon gauze. The crude nuclei or tissue homogenates were pre-digested with RNase A and T_1 (400 U/g tissue of each) at 37°C for 1 h. After adding proteinase K (85–170 U/g tissue), digestion was continued for an additional 2 h at 37°C and overnight at 4°C. Samples were transferred to the 14 ml vessels of the DNA extractor and extracted twice with 70% phenol/chloroform/water for 10 min at room temperature. Residual phenol was removed by a single extraction with chloroform (8 min). All phase separations were carried out at 60°C. DNA was precipitated with ethanol, dried for 1–2 h at room temperature and dissolved in TE buffer (1 mM EDTA in 10 mM Tris–HCl, pH 7.8). DNA concentrations were determined by reaction with diphenylamine (24), using standard curves generated with unmodified calf thymus DNA.

Quantitation of O^6-MEdG

O^6-MEdG was quantitated by an immuno-slot-blot assay using a rabbit antiserum raised against keyhole limpet haemocyanin conjugates of O^6-methylguanosine (NPZ-193; 1:8000) essentially as described earlier (23), with the following modifications: DNA (12 μg in 200 μl) was denatured for 10 min at room temperature with 200 μl of 100 mM NaOH, neutralized with 200 μl of 15% (v/v) acetic acid and mixed with 200 μl of 4 M ammonium acetate. An alkaline phosphatase conjugated goat-anti-rabbit IgG antibody was used as a second antibody. Bound antibodies were visualized with 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (0.136 mg/ml) and nitroblue tetrazolium chloride (0.33 mg/ml) in diethanolamine buffer (0.1 M Tris–HCl, 0.1 M NaCl, 25 mM diethanolamine, 2 mM $MgCl_2$, 1 μM $ZnCl_2$, pH 9.55) (25). Densitometric evaluation was performed at 530 nm using a Shimadzu Model CS-930 dual wavelength thin-layer chromatogram scanner in the zig-zag mode. Standard curves were generated with DNA which was methylated *in vitro* with methylnitrosourea and calibrated by HPLC with fluorescence detection (26). Peak heights were corrected for background binding to unmodified DNA and plotted against O^6-MEdG concentrations in a double log plot. The limit of quantitation was 1.5 μmol O^6-MEdG/mol deoxyguanosine.

Statistical analysis

Mean and standard deviations of duplicate determinations in animals assessed individually were established by one-way analysis of variance. The double-tailed Student's *t*-test was used to compare DNA methylation in rats receiving different alcoholic beverages. *P* values of 0.05 or less were considered significant.

Table I. Ethanol content of the alcohol beverages examined in this study

Beverage	Ethanol (g/l)
Pilsner beer (Germany)	39.8
White wine (France)	102.0
Red burgundy wine (France)	106.8
Sake (Japan)	150.7
Pear brandy (Switzerland)	329.8
Gin (Great Britain)	333.2
Commercially distilled calvados (France)	333.2
Scotch whisky (Scotland)	353.6
Farm-made calvados (France)	569.7

Results

The effects of a single oral dose of ethanol on the bioactivation of simultaneously administered NMBzA in oesophagus, lung and liver are shown in Figure 1. Animals received a single oral dose of NMBzA (2.5 mg/kg; 7.4 ml/kg) which was dissolved in tap water containing ethanol at concentrations ranging from 0 to 20% (v/v), i.e. the total amount of ethanol administered was 0–1.2 g/kg. Levels of O^6-MEdG were determined after a survival time of 3 h. In oesophagus, concentrations of O^6-MEdG increased from 15 μmol O^6-MEdG/mol dG in rats treated with 0.1% ethanol to 46 μmol/mol in animals given 20% ethanol. As shown in Figure 2, the log of O^6-MEdG concentrations in this tissue increased linearly with the log of dose for solutions containing 1–20% ethanol. The regression coefficient, calculated by the least-squares method, was 0.31 (r = 0.999), indicating that the relative increase in formation of O^6-MEdG in oesophageal DNA with respect to the amount of ethanol ingested was proportionally smaller at high doses. A dose-dependent increase in DNA methylation with increasing doses of ethanol was also observed in lung. In contrast to oesophagus, saturation at a plateau value of ~25 μmol/mol was achieved with 5% ethanol; the half-maximal effect was estimated to have been attained with 2.5% ethanol. In liver there was a marginal but statistically non-significant trend towards inhibition of DNA methylation with

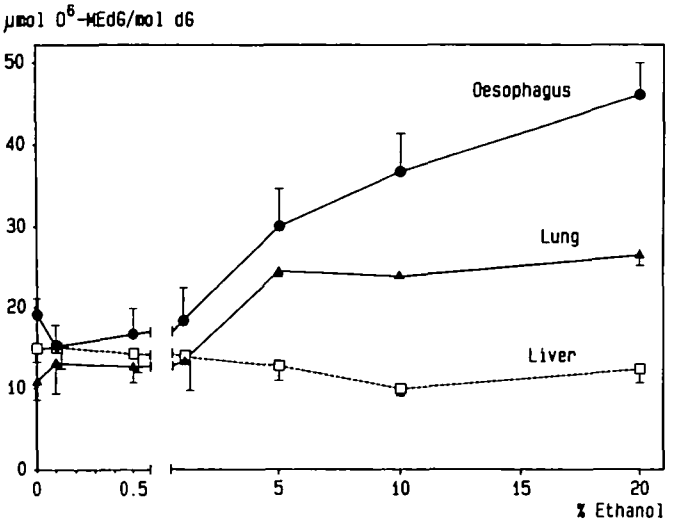

Fig. 1. Influence of concurrently administered ethanol on DNA methylation by NMBzA in rat oesophagus, lung and liver. Concentrations of O^6-MEdG were determined 3 h after a single dose by gavage of NMBzA (2.5 mg/kg body wt; 7.4 ml/kg) in tap water containing 0–20% (v/v) ethanol. Data represent mean ± SD of 4–6 determinations in pooled DNA from five animals.

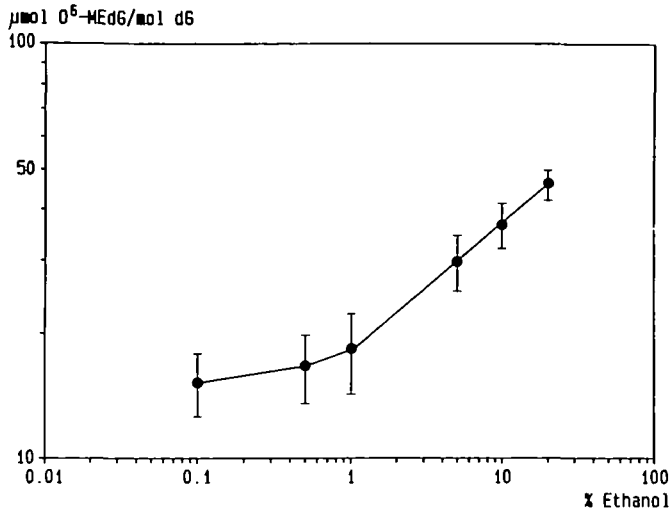

Fig. 2. Dose–response curve for the increase in oesophageal DNA methylation by NMBzA elicited by simultaneously administered ethanol. Data are taken from Figure 1.

Table II. Effect of various alcoholic beverages on the formation of O^6-MEdG from NMBzA in rat oesophagus[a]

Beverage	O^6-MEdG/dG	Increase (%)
Control		
Tap water	13.8 ± 1.7[b]	0
4% ethanol (v/v)		
Ethanol	25.8 ± 3.8	87
Pear brandy	20.7 ± 4 8	50
Sake	21.1 ± 3 5	53
Farm-made calvados	21.6 ± 2.7	56
Gin	22.2 ± 2.5	61
Scotch whisky	22.2 ± 3.2	61
White wine	27.9 ± 3.4	102
Pilsner beer	29.8 ± 2.5	116
Commercially distilled calvados	31.1 ± 3.6	125*
Red burgundy wine	36 2 ± 3.3	162**
20% ethanol (v/v)		
Ethanol	34.2 ± 4.2	148
Farm-made calvados	41.4 ± 1.9	200***

[a]Determined 3 h after a single intragastric dose of NMBzA (2.5 mg/kg; 7.4 ml/kg) in water, aqueous ethanol or alcoholic beverage (diluted to the indicated ethanol content with tap water).
[b]µmol O^6-MEdG/mol dG; mean ± SD of three to six determinations in pooled DNA from five animals.
The increase in O^6-MEdG levels was significantly larger than with an equivalent dose of aqueous ethanol: $^*P < 0.05$, $^{**}P \ll 0.001$ or $^{***}P < 0.01$.

increasing doses of ethanol. In nasal mucosa, initial formation of O^6-MEdG from NMBzA increased from 3.9 ± 1.7 µmol/mol dG in controls given tap water to 30.7 ± 1.1 µmol/mol with 4% ethanol ($n = 3$).

The extent of DNA methylation in oesophagus and liver of rats given NMBzA together with various alcoholic beverages was determined in a second experiment. In order to be able to detect enhancing as well as inhibitory effects on DNA methylation by congeners in the beverages beyond the modulation of NMBzA metabolism by ethanol, the beverages were diluted to a final alcohol concentration of 4% with tap water prior to administration. The results are summarized in Table II. Increases in levels of oesophageal O^6-MEdG elicited by pear brandy, Japanese sake, farm-made calvados, gin, Scotch whisky, white wine or Pilsner beer (+50% to +116%) were not significantly different from that of an equivalent dose of ethanol in tap water (+87%). In contrast, significantly higher increases in the formation of O^6-MEdG were obtained with commercially produced apple brandy (+125%, $P < 0.05$) and red wine (+162%, $P << 0.001$). Farm-made calvados was also tested at an ethanol concentration of 20%. An increase of 200% in oesophageal DNA methylation was observed, which was significantly larger ($P < 0.01$) than with pure ethanol (+148%). The effects of the alcoholic beverages on levels of hepatic O^6-MEdG were statistically non-significant (data not shown).

Discussion

In rats, first-pass clearance by the liver of small oral doses (<0.4 µmol/kg) of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine is reduced by a concurrent dose of 10 ml/kg body wt of 5% (v/v) ethanol, leading to increased metabolism in extrahepatic tissues (18). A similar shift in bioactivation from liver to extrahepatic tissues by comparable amounts of ethanol has also been observed with nitrosamines administered at doses more than two orders of magnitude higher (19,20,27). Ethanol is a competitive inhibitor of hepatic *N*-nitrosodimethylamine metabolism, with a K_i *in vitro* of 0.32–0.5 mM (17,18). Our results indicate that orally administered ethanol is a similarly potent modulator of nitrosamine metabolism *in vivo*. As shown in Figure 2, simultaneous administration of 7.4 ml/kg of 1% (v/v) ethanol, estimated to produce an initial concentration in blood of ~1.5 mM (18), was sufficient to increase oesophageal DNA methylation by a concurrent dose of 18 µmol/kg of NMBzA. It is interesting to note that though the increase in oesophageal DNA methylation was dose dependent for solutions containing 1–20% of ethanol, low doses produced a larger effect per gram of ethanol than did higher doses. Levels of O^6-MEdG in liver were close to the level of quantitation, with rather large variances obscuring the effects of ethanol on NMBzA bioactivation in this tissue. A slight, albeit statistically non-significant, trend towards decreases in DNA methylation with increasing doses of ethanol was observed, however, suggesting that an inter-organ shift in NMBzA metabolism from liver to lung and oesophagus would have been detected with more sensitive methods of adduct quantitation.

The large ethanol-mediated increase in the formation of O^6-MEdG from NMBzA in nasal cavity contrasts sharply with previous observations (20) of almost complete inhibition in this tissue of the bioactivation of two structurally related asymmetric nitrosamines, *N*-nitrosomethyl- and *N*-nitrosoethyl-n-butylamine, by a concomitant dose of ethanol. These observations provide evidence for the expression of organotypic cytochrome P450 isozymes with marked differences in susceptibility to inhibition by ethanol in addition to distinct substrate specificities for carcinogenic nitrosamines. Long-term carcinogenicity assays (reviewed in ref. 28) have suggested that for certain nitrosamines, organ-selective inhibition of bioactivation by coincident ethanol plays a predominant role in the modulation of tumorigenicity and organotropism. For example, in mice, co-administration of *N*-nitrosodimethylamine and ethanol reduced the incidence of hepatomas but led to the induction of olfactory neuroblastomas not seen in the absence of ethanol (12). Conversely, it has been reported that ethanol given after the carcinogen had no effect on tumour induction by some nitrosamines (29–31).

Epidemiological studies have revealed that the risk for

oesophageal cancer varies markedly with the type of beverage consumed and, in Western countries, is highest for apple-based beverages (1,2). We therefore also investigated whether alcoholic beverages differ in their effects on NMBzA bioactivation. In order to be able to detect a reduction as well as an increase in oesophageal levels of O^6-MEdG, the beverages were adjusted to a final alcohol content of 4% with tap water. The total amounts of beverage administered were equivalent to a person of 80 kg drinking 470 ml of beer, 180 ml of red or white wine, 120 ml of sake, 50 ml of pear brandy, gin, whisky or commercial brand calvados, or 30 ml of farm-made calvados—average-sized single servings. Under these conditions, commercially distilled calvados and red wine produced significant increases in oesophageal DNA methylation beyond the enhancement observed with an equivalent amount of aqueous ethanol, suggesting that these beverages contained congeners which by themselves or in synergism with ethanol induce an additional inter-organ shift in nitrosamine metabolism from liver to extrahepatic tissues. In contrast, pear brandy, sake, whisky, gin and farm-made calvados elicited increases in oesophageal DNA methylation which were marginally, though not significantly, lower than with the same dose of ethanol given in water. One class of congeners that could have caused this reduction in the effects of ethanol on NMBzA metabolism are long-branched-chain higher alcohols, flavour compounds found in most alcoholic beverages (32), which have been reported to be extremely potent inhibitors of nitrosamine bioactivation in hepatic and oesophageal microsomes (33).

Although the actual amounts of each beverage administered in this study roughly corresponded to typical amounts ingested during moderate social drinking, the individual beverages were diluted 1.1-fold (beer) to 10-fold (farm-made calvados) in order to achieve the same final ethanol concentration of 4%. In contrast to the results obtained with an ethanol content of 4%, farm-made calvados adjusted to 20% ethanol led to a significantly higher increase in oesophageal DNA methylation by NMBzA than aqueous ethanol. This suggests that the effects on nitrosamine metabolism upon ingestion of undiluted beverages, or of a larger single dose, may be greater than or different from those reported here. Furthermore, potentiation or modulation of the effects on the bioactivation of NMBzA observed with a single concomitant dose might occur with chronic exposure to alcoholic beverages as a result of intracellular accumulation of active compounds to levels significantly above those attained with a single dose.

We have previously shown that chemically induced oesophageal papillomas retain the capacity for nitrosamine bioactivation (34). Increased carcinogen metabolism during extended exposure to ethanol could, therefore, stimulate malignant progression by facilitating the accumulation of additional mutations in preneoplastic lesions. Chronic exposure to alcoholic beverages may exert an influence on tumorigenesis by a number of other mechanisms. Stimulation of cell proliferation by ethanol (35,36) and by 2-methylbutanol (33), a higher alcohol found predominantly in wine and distilled spirits (32), has been suggested to accelerate tumour formation from initiated cells. Ethanol is also an inducer of cytochrome P450 enzymes in liver (17) and extrahepatic tissues (16,37,38). Prolonged consumption of alcoholic beverages could similarly lead to enzyme induction in select tissues by as yet uncharacterized congeners, though this has not been demonstrated. The relative contributions of the various mechanisms to the increase in tumour incidence caused by dietary ethanol in humans remain to be elucidated. Thus, although we observed a significant increase in DNA methylation by NMBzA with red wine, the relative risk for oesophageal cancer in humans appears to be lower for wine than for other alcoholic beverages (1,2,7).

In conclusion, the present study demonstrates that ethanol is an effective modulator of the bioactivation of concurrently ingested nitrosamines at levels of alcohol intake that are equivalent to moderate social drinking, and that some widely consumed beverages contain congeners which amplify the effects of ethanol. In view of the strong synergism between drinking and smoking, an ethanol-mediated inter-organ shift in nitrosamine bioactivation could play an important role in the etiology of human cancer.

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